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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGB_X EFFECT

V. THE EFFECT OF HYPOXIA

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Normal RLM exposed to hypotonic media at 27^{o} rapidly lose their ability to carry out oxidative phosphorylation. However when the hypotonic exposure is carried out in the absence of oxygen, these RLM will maintain a high degree of oxidative phosphorylation. PGB_X added to these RLM has no effect on the oxidative phosphorylation reactions.

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INTRODUCTION

The therapeutic use of PGB_x (1, 2, 3, 4) in the treatment of human ischemic pathologies was suggested by successful animal experiments in a number of laboratories (5, 6, 7, 8, 9, 10, 11, 12). Before human trials may be attempted, it is necessary to first know the mechanism of the in vivo action of PGB_x. Towards this end studies have been underway in this laboratory to elucidate the in vitro mechanism of the PGB_x effect on phosphorylation activity of degraded RLM, in the hope that this would then lead to an understanding of the in vivo mechanisms of action.

In earlier reports Polis et al (3) and Angelakos et al (7, 8) indicated that the PGB_x effect can only be demonstrated with degraded RLM. In preceding reports of this series, we pointed out that the PGB_x effect could only be demonstrated with RLM exposed to modified State 4 Respiratory Conditions (13) i.e. nucleotides omitted. It was further reported that the PGB_x effect was dependent upon the composition of the State 4 Respiratory Conditions (14) and on the order of addition of PGB_x and RLM to the test system (15).

In this report we show that oxygen is an absolute requirement for the PGB_x effect. This oxygen requirement is over and above that required for oxidative phosphorylation reaction of RLM.

EXPERIMENTAL

Methods and Materials

PGB_x (Type II) (16) was synthesized as described previously (2, 3, 4) and assayed for its effect on the oxidative phosphorylation ability of degraded RLM by the method of Polis et al (2, 3, 4). The dissolved oxygen concentration was determined with a YSI Biological Oxygen Monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio).

Note: Abbreviations used in this report are: RLM, rat liver mitochondria; mS4RC, modified State 4 Respiratory Conditions; Pi, inorganic phosphate.

RESULTS

Stability of RLM exposed to anaerobic conditions

In the normal PGB_x *in vitro* assay system, RLM are incubated in the mS4RC mixture equilibrated with air during both the degradation stage and the phosphorylation stage. In this study we carried out the degradation of RLM in a deoxygenated medium, and since oxygen is an absolute requirement for oxidative phosphorylation, it was necessary then to re-equilibrate the deoxygenated medium with air prior to the addition of the reactants of the 2nd stage of the PGB_x assay. The experimental protocol is described in the following text.

TABLE I

The Composition of the Medium for the Demonstration of the PGB_x Effect on Mitochondrial Oxidative Phosphorylation

Order of Addition	Mitochondrial Degrading Medium	Reaction Mixture
Water	1.55 ml	1.55 ml
Phosphate Buffer $_pH$ 7.35	4.98 mM	4.55 mM
α-Ketoglutarate $_pH$ 7.35	14.93 mM	13.64 mM
$MgSO_4$	4.98 mM	4.55 mM
Aged Mitochondria	1.99 mg/ml	1.82 mg/ml
Sucrose*	5.97 mM	5.45 mM
EDTA*	0.010 mM	0.0009 mM
AMP	-----	2.27 mM
ADP	-----	2.27 mM
KCl	-----	45.45 mM
Bovine Serum Albumin	-----	0.68 mg/ml

Total Volume: 2.20 ml
Temperature: 28°

Degradation Time: 5-20 minutes
Reaction Time: 20 minutes

*Added with mitochondria

Table I lists the reactants of Stage 1 and Stage 2 of the _in vitro_ PGB_x assay system as well as the assay conditions. In this study, because of the size of the chambers of the Biological Monitor, the total volume was doubled. The degradation mixture (3.94 ml) was added to the Biological Oxygen Monitor Chamber and equilibrated to constant temperature (27^o). The oxygen sensor was then inserted in the chamber and lowered to about 1/2 cm above the liquid. A stream of nitrogen was directed just above the stirred liquid by means of a narrow gauge hypodermic needle fitted through the access slot of the sensor. At periodic intervals the gas flow was stopped, the sensor lowered below the liquid level to expell all gas bubbles and the dissolved oxygen determined. If the dissolved oxygen content was not zero, the sensor was raised above the liquid and the flow of nitrogen continued. This process was repeated until the mixture was completely deoxygenated. In preliminary tests the time required to deoxygenate the degradation medium was 1.5 minutes, however in this study a deoxygenation time of three minutes was used routinely to assure complete deoxygenation without the need to analyze the oxygen content for each test. The purpose of this was to maintain a constant volume of the degradation mixture since each time the sensor is lowered into the liquid some of the liquid is expelled through the access slot. RLM (8.0 mg) were added to the deoxygenated medium, the flow of nitrogen started and the solution stirred. In this way the RLM were exposed to a hypotonic medium under anaerobic conditions. At the end of the exposure time, the sensor was removed and the gases above the liquid replaced with air by applying a vacuum in the chamber. In preliminary tests it was found that the solution could be completely reoxygenated within ten seconds. Routinely 15 seconds reoxygenation time was used and the reagents of Stage 2 _in vitro_ PGB_x effect were then added and the reaction continued for 20 minutes. The Pi esterified was then measured as described previously (2, 3). With this short reoxygenation time no adverse effect on RLM phosphorylation activity was measurable.

Figure 1 shows the effect of exposing RLM to mS4RC under aerobic and anaerobic conditions. The results are plotted as the residual phosphorylation ability of RLM as a function of time of exposure to the hypotonic media. Curve X——X is the normal PGB assay curve, i.e. aerobic, while curve o —— o the assay curve obtained under anaerobic conditions. The phosphorylating ability of RLM exposed to aerobic conditions falls rapidly with time, and by eight minutes is completely void of all phosphorylating activity. This rate of loss of activity is a function of the age of RLM. Freshly isolated RLM still are capable of phosphorylation activity even after 20-30 minutes exposure. However the time required to reduce the phosphorylation activity is lessened as the RLM age. In contrast to the normal response, the phosphorylation activity of RLM exposed to hypotonic medium in the absence of oxygen was maintained at a consistently high level, even after 20 minutes exposure.

The effect of PGB_x on the phosphorylation activity of RLM exposed to hypotonic media under anaerobic and aerobic conditions were tested by carrying out the assays with varying amounts of PGB_x using a constant exposure time of five minutes. The results are plotted in Figure 2. As expected, the aerobic reaction gave the typical PGB_x-Type II biphasic response in that a maximum phosphorylation activity was found when RLM were treated with 2.27 to 9.09 μg PGB_x per ml of reaction (1.25 to 5.0 μg PGB_x/mg RLM). In contrast only minor changes were observed in the phosphorylation ability of RLM degraded under anaerobic conditions. With only a five minute exposure time, only a small amount of phosphorylation inhibition was observed (see 0 PGB_x concentration). At higher concentrations of PGB_x there was an improvement in phosphorylation activity. Most interesting was the finding that the highest concentration of PGB_x (20 μg/mg RLM), completely reduced the phosphorylation ability of aerobically degraded RLM to zero. Under anaerobic conditions similarly treated RLM functioned with a high degree of phosphorylation ability.

DISCUSSION

The *in vitro* PGB_x effect on rat liver mitochondrial oxidative phosphorylation was proposed by Polis *et al* (2, 3) to be an enhancement of the decreased phosphorylation ability of damaged RLM resulting from exposure to hypotonic media at 27°. This PGB_x effect was recently shown by Shmukler *et al* (14, 15) to take place only when RLM were exposed to degradative conditions in the presence of PGB_x. From these results it was concluded that PGB_x functions to stabilize RLM during exposure to degradative conditions. The results of the study reported here, support this mechanism. When RLM are exposed to hypotonic shock under anaerobic conditions, they still maintain a high degree of oxidative phosphorylation, suggesting that these RLM are not degraded. This conclusion is supported by the finding that when these RLM are pretreated with PGB_x according to the *in vitro* PGB_x assay system, no effect on phosphorylation was observed. This is similar to that observed when undegraded isolated RLM are treated with PGB_x.

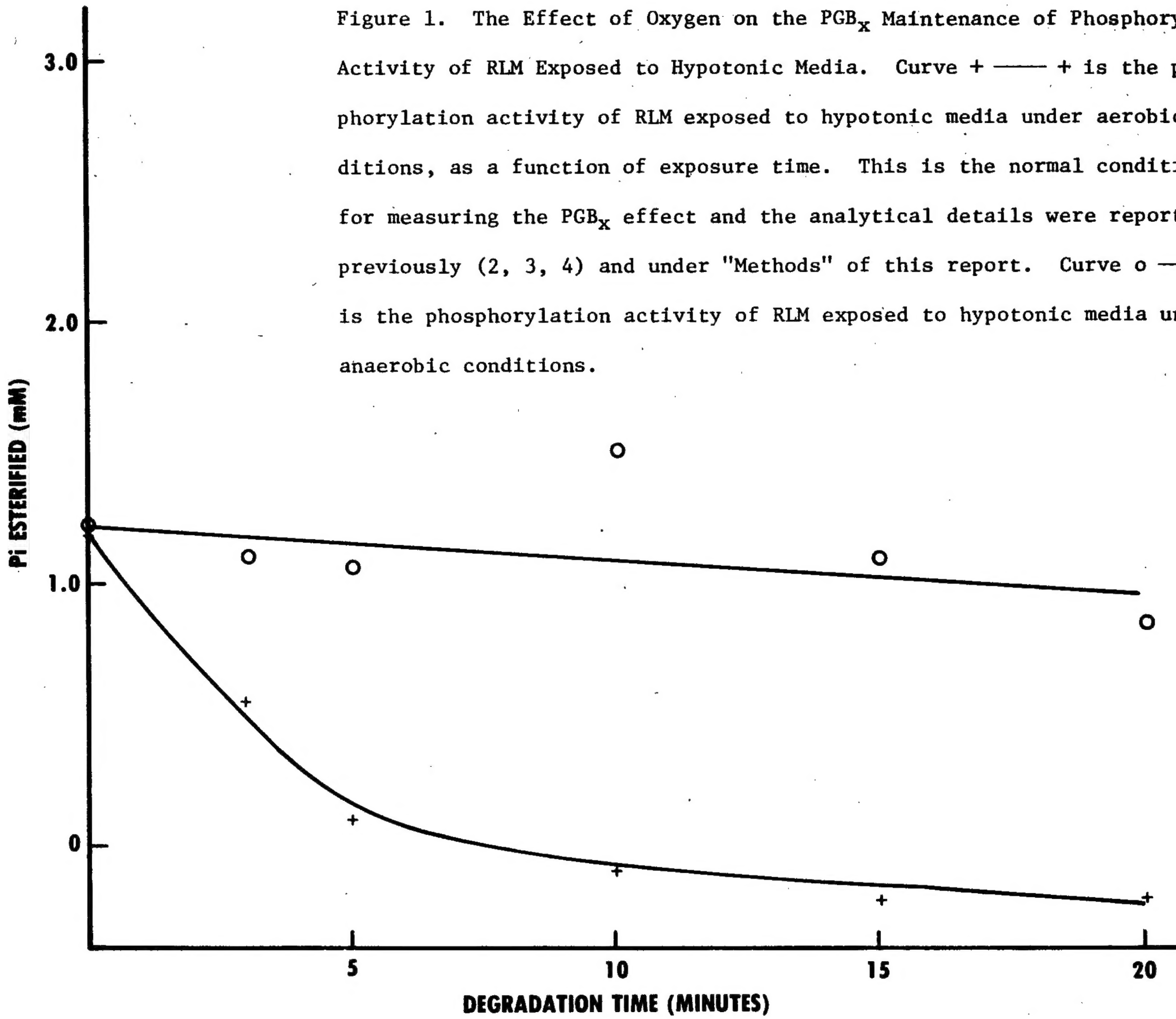

Figure 1. The Effect of Oxygen on the PGB_x Maintenance of Phosphorylation Activity of RLM Exposed to Hypotonic Media. Curve + —— + is the phosphorylation activity of RLM exposed to hypotonic media under aerobic conditions, as a function of exposure time. This is the normal conditions for measuring the PGB_x effect and the analytical details were reported previously (2, 3, 4) and under "Methods" of this report. Curve o —— o is the phosphorylation activity of RLM exposed to hypotonic media under anaerobic conditions.

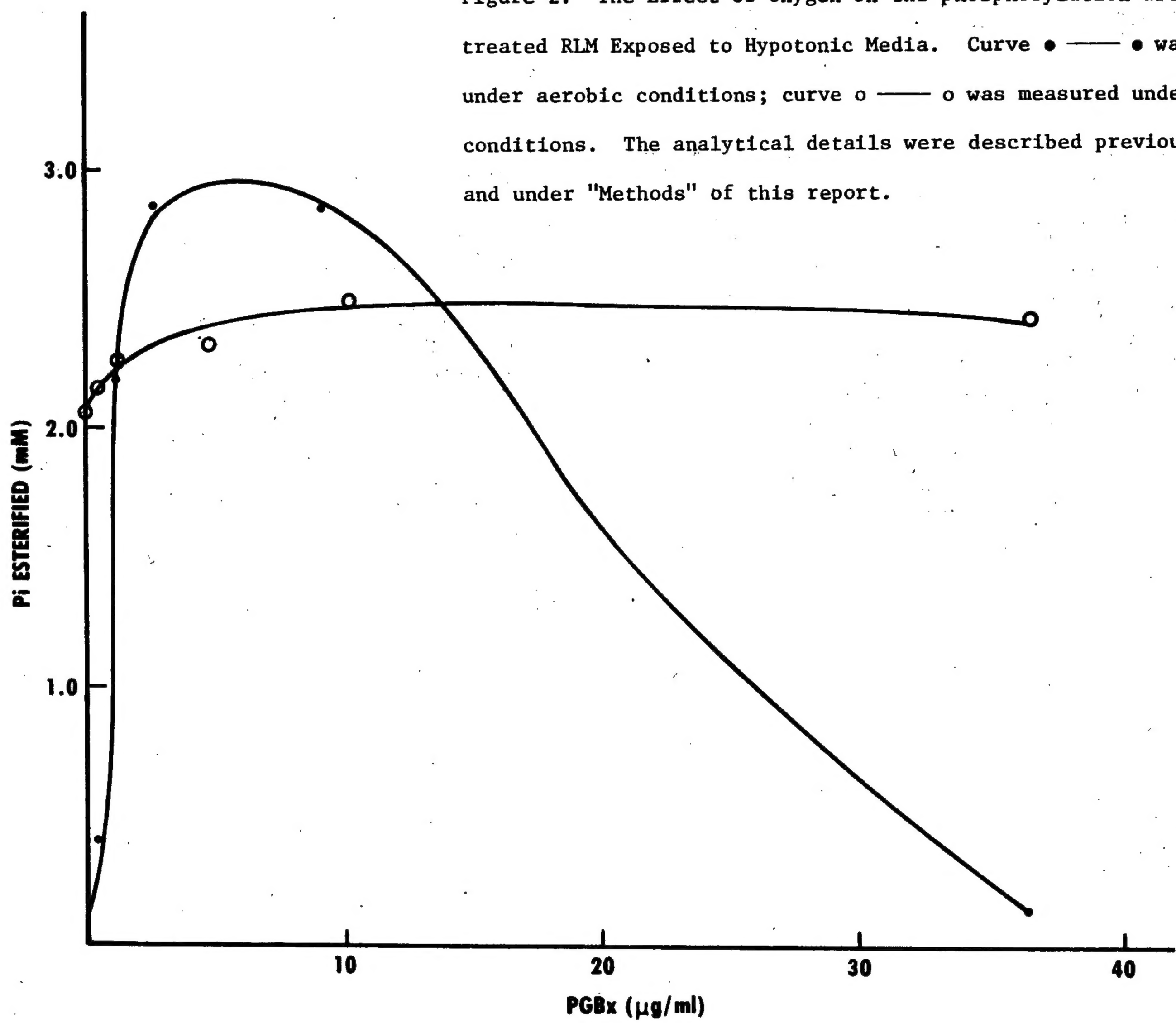

Figure 2. The Effect of Oxygen on the phosphorylation ability of PGB_x-treated RLM Exposed to Hypotonic Media. Curve ● —— ● was measured under aerobic conditions; curve o —— o was measured under anaerobic conditions. The analytical details were described previously (2, 3, 4) and under "Methods" of this report.

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